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Mutants for rice storage proteins

1. Screening of mutants for rice storage proteins of protein bodies in the starchy endosperm

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Summary. To obtain genetic materials to breed qualitatively improved rice storage proteins, we screened about 3,000 mutant lines induced by the treatment of rice fertilized egg cell with N-methyl-N-nitrosourea (MNU). The screening was performed by comparing the profiles of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with that of the original variety, Kinmaze, especially focussing on the changes in polypeptides present in two kinds of protein bodies, PB-I and PB-II. We selected 17 mutant lines and classified them into 4 types on the basis of variations of the relative contents of the polypeptides. Determination of extracted protein in the starchy endosperm of the mutants revealed changes in the content of prolamin and glutelin but not globulin. In some mutants there was marked accumulation of 57 kDa polypeptide concomitant with the remarkable reduction of glutelin subunits. Treatment of the fertilized egg cell with MNU was found to be an effective method to induce mutations for storage proteins in protein bodies of rice.

Key words: Endosperm – Mutant – *Oryza sativa* L. – Protein body – Storage protein

Introduction

A great deal of work has been done on the genetic research and improvement of the seed storage protein, and mutants for seed storage protein have been reported to occur in maize (Mertz et al. 1964; Nelson et al. 1965), barley (Munck et al. 1970; Shewry et al. 1978), wheat (Payne et al. 1981), soybean (Kitamura and Kaizuma 1981) and oat (Robert et al. 1983). In rice, the improvement of storage proteins has been reported only with regard to increasing the total content of storage proteins (Beachell et al. 1972; Higashi et al. 1974; Kambayashi et al. 1984; Osone and Takagi 1970) and the lysine content in the storage proteins (Shin et al. 1977), but the qualitative improvement of rice proteins has yet to be investigated.

The major storage protein in rice starchy endosperm is an acid and/or alkaline soluble protein (glutelin), termed oryzenin, which accounts for 80% of total protein in the endosperm (Juliano 1972). Salt and alcohol soluble proteins (globulin and prolamin) are present in relatively low amounts in the rice endosperm (Cagampang et al. 1966; Juliano 1972), although they are the major storage proteins in legumes and other cereals (Pernollet and Mossé 1983). In some cereals, the storage proteins are deposited in one kind of protein body in the endosperm (Pernollet and Mossé 1983). In the case of rice, however, storage proteins are accumulated separately in two different kinds of protein bodies. Rice glutelin is stored in protein body type II (PB-II), which is a uniformly electron dense structure 2–3 μm in diameter (Tanaka et al. 1980), and rice prolamin is deposited in protein body type I (PB-I), which is a spherical proteinaceous particle of lamellar structure 1–2 μm in diameter (Tanaka et al. 1980; Ogawa et al. 1987). Studies on the characterization (Zhao et al. 1983; Wen and Luthe 1985; Sarker et al. 1986) and localization of rice storage proteins (Tanaka et al. 1980; Yamagata et al. 1982; Yamagata and Tanaka 1986; Krishnan et al. 1986; Ogawa et al. 1987) have confirmed that rice glutelin is composed of two kinds of acidic and basic subunits with MWs of about 40,000 and 20,000, respectively, and exists in PB-II, while rice prolamin consists of one major polypeptide component with MW 13,000, and is deposited in PB-I.

Important evidence on the nutritional value of rice storage proteins has been reported by Tanaka et al. (1975a), who confirmed by electron microscopic observation that the spherical proteinaceous particles (fecal protein particles; FPP) was observed in fresh human feces (Tanaka et al. 1975b). Although this suggests that PB-I is nutritionally inferior to PB-II, recently we found that PB-I accounts for about 20%–25% of total rice endosperm proteins (Ogawa et al. 1987). Hence, from the standpoint of the qualitative improvement of rice protein, the proteins present in PB-I should not be neglected since they form a rather important part of the protein source in rice.

Based upon the evidence obtained so far, the direction of breeding for qualitative improvement of rice storage proteins should be focussed not only on increasing the content of proteins present in PB-II, but also on reducing the proteins in PB-I. Moreover, emphasis should be placed on changing the physicochemical properties of PB-I to increase its digestibility.

Regarding mutation techniques, we have found that treatment of the rice fertilized egg cell with N-methyl-N-nitrosourea (MNU) was highly effective for inducing various kinds of mutations (Satoh and Omura 1979). Recently, we have found various endosperm mutants (Satoh and Omura 1981; Omura and Satoh 1984) including high amylose mutants like the amylose extender of maize (Yano et al. 1985) and high oil content mutants (Yoon 1984; Matsuo et al. 1987). Thus, there is ample hope to effectively obtain mutants for improved storage proteins by using the present mutation methods.

In this paper, we have screened mutant lines for storage proteins from MNU-induced mutant lines focussing on proteins in PB-I and PB-II by using SDS-PAGE to characterize those proteins in mutants. The significance of these findings for genetic resource of those mutants is also discussed.

Materials and methods

Plants

About 3,000 mutant lines used in this experiment were stocked at the Plant Breeding Laboratory, Faculty of Agriculture, Kyushu University, Japan. These lines were produced by treatment of the rice (*Oryza sativa* L. cv Kinmaze) fertilized egg cell with MNU as reported elsewhere (Satoh and Omura 1979). Rice plants were cultured by water culture in 1/5,000 a Wagner pot containing nutrients (Yoshida et al. 1976) in a glass house at the Research Institute for Food Science, Kyoto University.

SDS-PAGE

Extracted storage proteins of mutant lines were electrophoresed using the discontinuous buffer system of Laemmli (1970) on a slab gel containing 14% or a linear 15%–30% acrylamide concentration gradient.

Screening of mutants

Proteins were extracted from one grain for each mutant line. Each grain was grounded by motor and pestle in 0.5 ml Solvent A (50 m*M* KH_2PO_4-NaOH, pH 6.8, containing 8 *M* urea, 4% SDS, 20% glycerin and 5% 2-mercaptoethanol), and the homogenate was sonicated for a few minutes. After centrifugation (15,000 rpm, for 5 min) 10 µl of the supernatant was used for a SDS-PAGE. Screening of mutants for storage proteins was done by comparing the SDS-PAGE profiles with that of the original variety, Kinmaze. Selection of mutants for storage proteins by SDS-PAGE analysis was repeated several times.

Characterization of polypeptide composition in mutants

Original variety (Kinmaze), CM 21, CM 1675, CM 1787 and CM 1834 were used in this experiment. Forty mg of powder of polished rice grains were homogenized in 0.5 ml of 50 m*M* KH_2PO_4-NaOH (pH 6.8) solution containing 0.5 *M* NaCl (Solvent B) and centrifuged and the supernatant discarded. One ml of Solvent A was added to the precipitate, which was sonicated for a few minutes, and then centrifuged. The supernatant (I) (20 µl) was applied on a 15%–30% gradient gel and electrophoresed as described before. Each lane of the gel was scanned at 535 nm on Beckman DU-8 Spectrometer to determine the staining intensities of the individual polypeptide.

Two-dimensional gel electrophoresis

The supernatant (I) was dialyzed against H_2O at 4 °C, freezed, thawed and centrifuged at 10,000 × *g* for 10 min. The precipitate was dissolved in the solution (O'Farrell 1975) and subjected to two dimensional gel electrophoresis, which was conducted according to the procedure of Wall et al. (1984). The protein samples were absorbed into filter paper strips and fixed on the gel near the anode wick. Electrophoresis was performed with circulating water (5 °C) at 1.6 W constant power for 14 h using a LKB 2117 Multiphor system. After electrophoresis in the first dimension, the gels were cut in 0.5 mm strips and immersed in 10 ml of solvent A at 37 °C for 1 h. The gels were then loaded on to 14% acrylamide gel and electrophoresis was done at 120 V for 6 h.

Determination of proteins extracted by solvents

Proteins from Kinmaze and mutant lines were extracted sequentially by several solvents. Solvent B (1.0 ml) for extraction of albumin-globulin was added to 100 mg powder of polished rice in a centrifugation tube. The suspension was sonicated for a few minutes on ice and centrifuged. The procedure was repeated three times. The supernatant was placed in a test tube. One ml of Solvent C (60% n-propanol containing 1 m*M* EDTA-2Na) for extraction of prolamin was added to the precipitate, and the extraction procedure was done. Finally, glutelin was extracted with 1.0 ml of Solvent D (1% lactic acid containing 1 m*M* EDTA-2Na). The albumin-globulin and glutelin fractions were filled up to 4 ml with distilled water, while n-propanol in prolamin fraction was evaporated in a drying box at 80 °C and filled up to 4 ml with distilled water. Thirty three µl of 2% deoxycholic acid was added to each fraction and vortexed. After 15 min, 1.3 ml of 24% trichloroacetic acid was added to each of them and allowed to stand overnight at 4 °C. They were centrifuged at 3,000 rpm at 2 °C for 15 min, and the supernatant was discarded. One ml of 1 N NaOH was added to the precipitate to solubilize proteins. The protein content of each fraction was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

Results and discussion

After screening about 3,000 mutant lines, we selected 17 mutants which showed marked differences in the concentration of polypeptides occurring in PB-I and PB-II, compared to that of Kinmaze (Fig. 1). The staining intensities of the individual polypeptide bands were assessed in relation to Kinmaze and the mutants were classified into four types, tentatively named types A, B, C and D. Types A and B were characterized by low and high intensities of the 10 kDa polypeptide band, respectively. Type C showed a low intensity of the 13 kDa polypeptide band. Type D showed a high intensity of the 57 kDa polypeptide band. The number of mutants for types A, B, C and D were 1, 1, 9 and 6, respectively. Types C and D are found frequently among MNU-induced mutants. These two types have been also found in native rice varieties (Shiraishi et al. 1986). On the other hand,

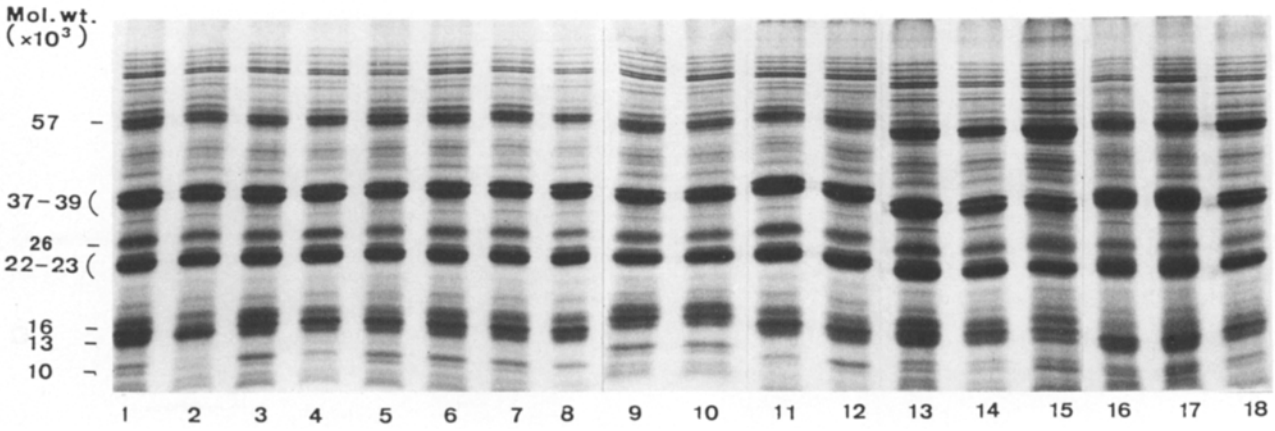

Fig. 1. SDS-PAGE analysis of the storage proteins in original variety and the screened mutants. *1* Original variety, Kinmaze; *2* CM 1675; *3* CM 1834; *4* CM 21; *5* CM 497; *6* CM 500; *7* CM 561; *8* CM 1257; *9* CM 575; *10* CM 1208; *11* CM 2000; *12* CM 2145; *13* CM 615; *14* CM 935; *15* CM 1787; *16* EM 25; *17* EM 61; *18* EM 305. *2* Mutant type A; *3* Mutant type B; *4–12* Mutant type C; *13–18* Mutant type D

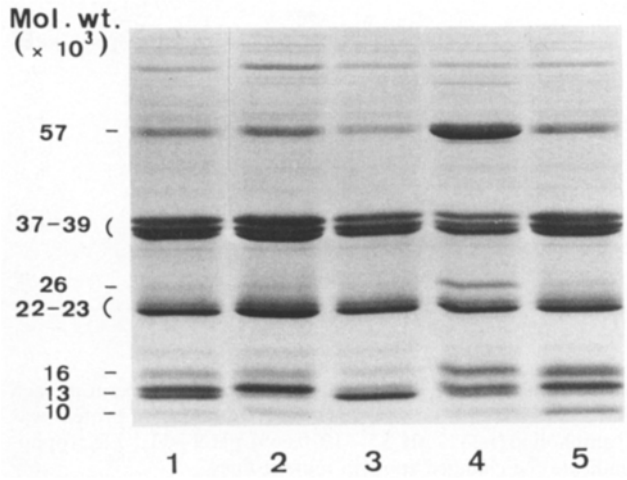

Fig. 2. SDS-PAGE analysis of salt insoluble proteins extracted from the starchy endosperm of each mutant type. *1* Kinmaze; *2* CM 21 (13b-L); *3* CM 1675 (10/13a-L); *4* CM 1787 (57-H); *5* CM 1834 (10/16-H)

Table 1. Relative contents of individual polypeptides in the starchy endosperm of mutants, calculated from the relative intensities which were determined by scanning the electrophoregrams of polypeptides separated on SDS-PAGE as shown in Fig. 2 by using a densitometer, values are presented as percentages

Line (Type of mutant)	Relative contents of individual polypeptides (%)						
	Molecular size (kDa)						
	57	37–39	26	22–23	16	13	10
Kinmaze	9.3	37.8	–	28.2	7.0	15.9	1.8
CM 21 (13b-L)	7.8	41.6	–	34.0	4.0	10.2	2.4
CM 1675 (10/13a-L)	9.0	40.8	–	30.8	2.9	16.5	–
CM 1787 (57-H)	35.2	24.7	5.7	16.2	8.7	9.2	0.2
CM 1834 (10/16-H)	9.1	40.0	–	24.9	10.0	11.1	5.1

the frequency of types A and B was quite low. We selected the mutant lines of CM 1675 (type A), CM 1834 (type B), CM 21 (type C) and CM 1787 (type D) for use in further experiments.

Recently, Ogawa et al. (1987) showed the diversity of protein constituents in PB-I purified from rice grains and confirmed that the proteins in PB-I are composed of four components such as 10 kDa, 13 a (a larger component of 13 kDa), 13 b (a smaller component of 13 kDa) and 16 kDa polypeptides that differ in their solubility to alcohol. To affirm the differences in the relative contents of individual polypeptides in each mutant, we analysed the proteins in mutants after removal of salt soluble proteins by electrophoresis in SDS-PAGE on a slab gel containing 15%–30% linear acrylamide concentration gradient (Fig. 2), and determined the relative intensities of individual polypeptides of each mutant by using a densitometer. The characteristic SDS-PAGE profiles of storage proteins in mutants observed in the first screening step were verified. It was further revealed that there is a distinct variation in the intensities of bands of the 13 a and 13 b polypeptides. In Kinmaze and CM 1787, both intensities were almost equal, but in CM 21 and CM 1834, the intensity of the 13 b polypeptide band was weaker than that of the 13 a polypeptide band, while the pattern of intensities was reversed in CM 1675. Table 1 shows the relative contents of individual polypeptides in Kinmaze and the mutants, which was calculated from the intensities of individual polypeptide bands assessed by the densitometric findings of Fig. 2. In this procedure,

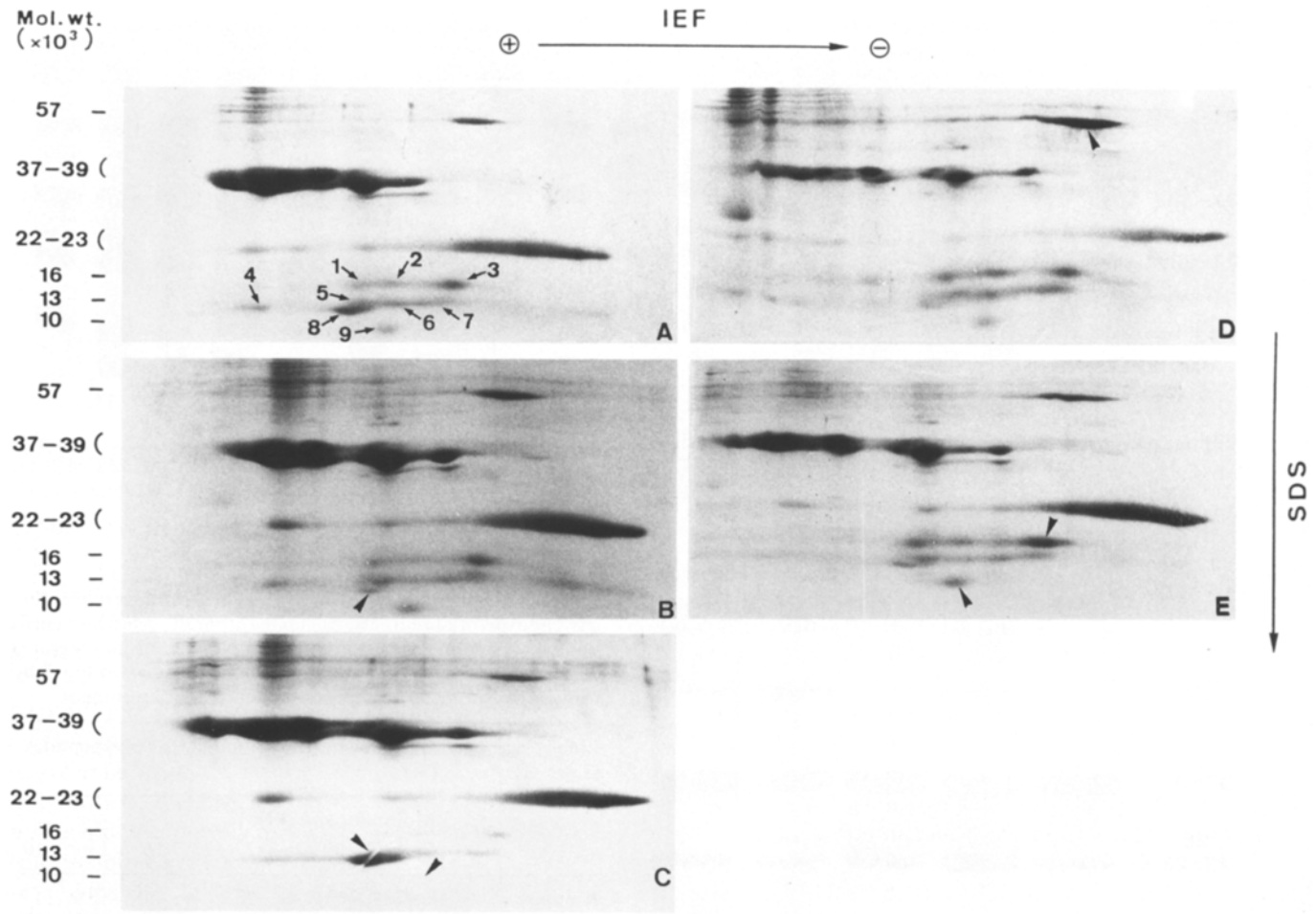

Fig. 3 A–E. Two-dimensional gel electrophoretic analysis of salt insoluble proteins extracted from the starchy endosperm of each mutant. **A** Kinmaze; **B** CM 21 (13b-L); **C** CM 1675 (10/13a-L); **D** CM 1787 (57-H); **E** CM 1834 (10/16-H). One mg of protein was applied on the first dimension (IEF). The IEF gels contained 2% (v/v) ampholine (1.34% pH 3.5–10, 0.66% pH 9–11). Electrophoresis in the second dimension was done by SDS-PAGE. *Arrows* (▲) indicate the changed spots in mutant lines

the 13 a and 13 b polypeptides were not densitometrically distinguished from each other. In CM 21, the relative content of 13 kDa polypeptide decreased in comparison with that of Kinmaze, showing that this reduction may be dependent on the decrease in the content of the 13 b polypeptide. In CM 1675, the content of the 13 kDa polypeptide was similar to that of Kinmaze, whereas the 10 kDa polypeptide was hardly distinguishable. Since the relative content of the 13 kDa polypeptide is similar to that of Kinmaze, the decrease of the 13 a polypeptide in CM 1675 (Fig. 2) is compensated by the increase of the 13 b polypeptide. In CM 1834, the 10 kDa polypeptide apparently increased and also the relative content of the 16 kDa polypeptide was higher than that of Kinmaze. The difference between three mutants (CM 21, CM 1675 and CM 1834) and Kinmaze was small in terms of the relative content of the 22–23 and 37–39 kDa polypeptides. In CM 1787, the relative content of the 57 kDa polypeptide increased remarkably in comparison with that of Kinmaze and other mutants. By contrast, the 22–23, 37–39 kDa and lower molecular weight polypeptides, except for the 16 kDa polypeptide, were reduced remarkably, suggesting that the accumulation of proteins in PB-I and PB-II is partly obstructed. On the basis of the characteristics of polypeptide compositions in mutants, we propose that types A, B, C and D be descriptively named 10/13 a-L, 10/16-H, 13 b-L and 57-H, respectively.

The polypeptide compositions of storage proteins in mutant lines and Kinmaze were investigated in detail by two-dimensional gel electrophoresis. The protein of 1 mg per line was used because less than 1 mg of protein did not give clear spots of a group of the 10–16 kDa polypeptides. The electrophoretic patterns of a group of the 22–23 kDa and 37–39 kDa polypeptides were similar to those obtained by Wen and Luthe (1985). In high molecular weight (22–57 kDa) regions, the spots of the 57 kDa polypeptide in CM 1787 enlarged to that of Kinmaze, while 22–39 kDa polypeptides were reduced (Fig. 3 D).

There were differences in electrophoretic patterns in low molecular weight (10–16 kDa) regions between the other lines. In Kinmaze, the 10–16 kDa polypeptides were separated into nine spots (Fig. 3 A). It was revealed that the 16 kDa and 13 a polypeptides consisted of more than 3 components (16 kDa, spots 1–3; 13 a, spots 4–7). On the other hand, the 13 b and 10 kDa polypeptides were separated into only one component (13 b, spot 8; 10 kDa, spot 9). In CM 21 (Fig. 3 B), spot 8 was quite faint and the intensity of other spots was similar to those of Kinmaze. In CM 1675 (Fig. 3 C), spot 9 was missing and conversely the intensity of spot 8 was stronger with comparison to Kinmaze and other mutants, and the other spots were faint. In CM 1834 (Fig. 3 E), the pattern was similar to that of CM 21, except for spot 3, which had the strongest intensity. Two-dimensional gel electrophoresis confirmed that the changes in electrophoretic pattern observed in SDS-PAGE (Fig. 2) is due to increase and/or decrease in 10 kDa, 13 b and one component of 16 kDa polypeptides.

Osborn's classification (1924) of proteins based on solubility has been widely used for the determination of protein composition in cereals. Table 2 shows the content of protein extracted with several kinds of solvent from rice starchy endosperm. Although there was no large difference in the albumin-globulin content of the mutants (1.01–1.34 mg) compared to Kinmaze (1.16 mg), there were differences in the prolamin and glutelin fraction. The prolamin content of CM 1675 was about twice that of Kinmaze and CM 1787 was about half. In the case of CM 1675, the 10 and 16 kDa polypeptides were almost absent and the 13 b polypeptide was remarkably increased (Fig. 3). This leads us to speculate that the increase of prolamin content in CM 1675 is the result of the increase of the 13 b polypeptide, in view of the findings in Table 2. In CM 21 and CM 1834, there was no remarkable reduction of prolamin, although the relative content of the 13 b polypeptide decreased, judging from their SDS-PAGE profiles (Figs. 2 and 3). It seemed that there may have been an increase in other kinds of alcohol soluble prolamin in PB-I, instead of a decrease in the 13 b polypeptide. Glutelin content of CM 1834 and CM 21 were 5.81 mg and 7.37 mg, respectively, which were higher than that of Kinmaze (4.74 mg). On the other hand, the glutelin content of CM 1675 and CM 1787 were lower than that of Kinmaze. Judging from the fact that prolamin is a major protein of PB-I (Ogawa et al. 1987) while glutelin is a major protein of PB-II (Tanaka et al. 1980), it is suggested that the number of PB-II in the cell or the absolute protein content of PB-II in CM 21 and CM 1834 are higher than that of Kinmaze. Therefore, CM 21 and CM 1834 may be regarded as high glutelin type mutants. It is of interest to note the remarkable reduction in glutelin content in CM 1787 that occurs along with the marked increase in 57 kDa polypeptide content. Since the subunit groups of glutelin, 22–23 kDa and 37–39 kDa polypeptides are formed through post-translational modification of the 57 kDa polypeptide (Yamagata et al. 1982, Yamagata and Tanaka 1986, Sarker et al. 1986), CM 1787 may be important to resolve the rice glutelin biosynthesis mechanism.

Table 2. The content of proteins extracted from the starchy endosperm of mutants. Proteins were extracted sequentially with several kinds of solvents as described in Materials and Methods. Proteins in each extracted fraction were determined by the Lowry method (Lowry et al. 1951)

Protein fraction	Protein content (mg/100 mg polished grain)				
	Kinmaze	CM 21	CM 1675	CM 1787	CM 1834
Albumin-Globulin	1.16	1.20	1.01	1.34	1.25
Prolamin	0.57	0.70	0.95	0.32	0.56
Glutelin	4.74	7.37	4.16	3.47	5.81
Total	6.47	9.27	6.12	5.13	7.62

The evidence indicates that high glutelin type mutants such as CM 21 and CM 1834 may be promising genetic materials for breeding of qualitatively improved rice proteins. Also, mutants such as CM 21, CM 1675 and CM 1834, which vary in their polypeptide composition of PB-I, may shed light on the genetic regulation of prolamin biosynthesis in PB-I. Further, CM 1787 could possibly be used for biochemical and genetical studies on the regulation, biosynthesis and accumulation mechanism of rice glutelin.

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